



**PATENT**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re: Patent application of :  
Alan Gewirtz :  
 : Group Art Unit: 1635  
Serial No.: 09/993,183 :  
 :  
Filed: November 14, 2001 : Examiner: Jon B. Ashen  
 :  
For: POST-TRANSCRIPTIONAL GENE SILENCING :  
BY RNAi IN MAMMALIAN CELLS : Conf. No. 6995  
 :

**DECLARATION OF DR. ALAN M. GEWIRTZ under 37 C.F.R. § 1.132**

**Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450**

**Sir:**

I, Alan M. Gewirtz, declare that:

1. I am the inventor of the invention disclosed and claimed in U.S. Patent Application Serial No. 09/993,183, claiming for the first time that it is possible to disrupt gene expression at the mRNA level in a target gene in a *human* cell by providing to that human cell, small interfering double stranded RNA (dsRNA) guide sequences that are homologous to a portion of the target gene, such that RNA interference (RNAi) of the target gene is induced, and I have provided evidence demonstrating that effect in several human cell lines.

2. To facilitate submission of this Declaration, I have not attached a copy of my *curriculum vitae*. Should my c.v. become required, I will produce it upon request. Briefly, however, my career has been devoted to studies of normal and malignant human hematopoiesis, translational strategies for silencing gene expression, and generally, to ways of translating basic scientific discoveries into clinically useful therapies for patients with disorders of blood cell formation. I am currently Professor of Internal Medicine and Pathology-Laboratory Medicine at the University of Pennsylvania School of Medicine, Division of Hematology/Oncology, and hold a number of faculty appointments at the University, including Leader of the Hematologic Malignancies Program in the Cancer Center,

and Member in the Cell and Molecular Biology, and the Pharmacology Graduate Groups. I hold an M.D. degree and an M.A. in microbiology from the State University of New York at Buffalo. In 1976, I began an Internship and Residency at Mt. Sinai Hospital in New York City which I completed in 1979. I then accepted Fellowships in both Hematology and Oncology at Yale University, School of Medicine, in New Haven, Connecticut which were completed in 1982. I am Board Certified in both Specialties. Upon completion of my fellowship training I received an Instructor appointment in the Dept. of Medicine at Yale which I resigned after one year to accept a position as Assistant Professor of Medicine and Thrombosis Research at Temple University in Philadelphia. I remained at Temple University through 1990 at which time I moved to the University of Pennsylvania to assume the position of Associate Professor (with academic tenure) in the Departments of Pathology and Medicine. I have remained at Penn ever since. Presently, I am hold the titles of Professor of Medicine, and Pathology and Laboratory Medicine, and Leader of the Hematologic Malignances Program at the University of Pennsylvania Cancer Center. I am also a full Member of the Leonard and Madelyn Abramson Family Cancer Research Institute. I was elected to the American Society of Clinical Investigation in 1990, and I have been honored with several awards, including the Scientific Achievement Award from the American Cancer Society; the Doris Duke Distinguished Clinical Scientist Award for Excellence in Bench to Bedside Research, William Osler Award for Patient Oriented Research (Univ. of PA); and recently, I was installed as the first C. Willard Robinson Professor of Hematology/Oncology at the University of Pennsylvania School of Medicine. I have served on numerous ad hoc NIH and foreign government (Canada, Switzerland, United Kingdom) review panels and served full terms on the NIH Experimental Therapeutics Study Section-I, and the Hematology Study Section of the Veterans Administration. I am a member of several editorial boards for specialty journals concerned with human stem cells, hematopoiesis and gene therapy, including Nucleic Acids Research and the Journal of Clinical Oncology. In addition, I recently chaired the Medical/Scientific Advisory Committee of the Leukemia and Lymphoma Society and I currently serve on its newly formed Board of Directors.

3. I have read all of the communications from the U.S. Patent and Trademark Office (PTO) relating to the above-identified application, and I have participated in the Response process for each Office Action. I was also an active participant at the in-person Examiner's

Interview for this application on July 18, 2005. Consequently, having talked to Examiners Ashen and Wang at length on the subject of my invention and having discussed with them their comments made in the Office Action dated May 23, 2005, I am well qualified to address their concerns and explain why my discovery of the inhibitory effect of RNAi on a target gene in a *human* cell, based upon actual testing in my laboratory initiated prior to Fall 2000, was not, and could not have been, anticipated, predicted or suggested by any prior teachings in the art.

4. Claims 1, 2, 5, 7-9, 11, 21-27 are pending in the application. Claims 1 and 22 are independent claims, and all other pending claims are directly or indirectly dependent upon either claim 1 or claim 22. As presently amended, claims 1 and 22 read as follows:

1. A method for disrupting target gene expression at the mRNA level in a human cell, wherein the method comprises initiating RNA interference (RNAi) *in vitro* by exposing the cell to a double stranded RNA (dsRNA) homologous to the target gene, wherein the dsRNA consists essentially of two complementary linearized strands of RNA, the transcription of each is independently controlled to generate paired RNAs of defined length.

22. A method for disrupting target gene expression *in vitro* at the mRNA level in a human cell, wherein the method comprises providing small interfering RNA guide sequences which are homologous to a portion of the target gene, such that RNAi of the target gene is induced.

5. It is my understanding that according to the Examiner in the Office Action dated May 23, 2005, paragraph 8, the pending claims are rejected under 35 U.S.C. 102(e) as anticipated by Fire *et al.* (US Pat. No. 6,506,559) because the Fire patent discloses “a method for inhibiting expression of a target gene using double stranded RNA to induce RNAi in a cell *in vitro* (col. 26, claim 1) wherein the cell is from an animal (col. 26, claim 6).” In making this argument, the Examiner states that “Fire *et al.* disclose that the cell with the target gene may be derived from or contained in any organism (col. 8, line 13-14) and that examples of vertebrate animals include mammals and human (col. 8, lines 35-37).”

6. In arguments of record, I have, through my counsel, argued that such a rejection is improper and incorrect. In fact, at the time of the ‘559 invention by Fire *et al.* (U.S. effective filing date claimed was December 23, 1997, actual US filing date was December 18, 1998), he and his co-inventors knew that the claimed method of using dsRNA inhibited expression only of a target gene in cells of an invertebrate embryo, *C. elegans*. As has been previously pointed out in the present record, in later publications Fire acknowledged that he did not actually believe that

his claimed method would actually work in a human cell. Others agreed that, at the time, there were many reasons for one skilled in the art to doubt that Fire's method could or would be effective, as claimed in the '559 patent, in a human cell. To suggest that Fire's specification, containing data showing *only* the effect of dsRNA in embryonic invertebrate cells, would suggest to one of ordinary skill at that time, that the method also taught how to inhibit expression of a target gene in a human cell, is to read far more into the '559 patent than was actually in the inventor's possession at the time of his invention or that he could have known. This is especially true given Fire's own subsequently published words questioning the effectiveness of his invention in a mammalian cell.

7. Fire's claim 1 is, in fact, silent on the cells for which his method is effective, although dependent claims specify an "animal" - without saying vertebrate or non-vertebrate, and subsequently clarifies that the cells are from a plant, invertebrate and nematode. While the '559 specification states at col. 8, line 16, that "the animal *may* be a vertebrate or invertebrate" (emphasis added), no other statement teaching or example in the entire application, other than at column 8, addresses any dsRNA response in a cell taken from a "vertebrate," or specifically from a human. Thus, the boilerplate lists included in the patent specification were merely wishful-thinking by Fire when the '559 specification was drafted. Evidence shows that Fire had no idea whether the system would or could operate in any cell other than an embryonic *C. elegans* cell, and in fact, there was strong evidence at the time to show why it would not operate in vertebrate cells.

8. Prior to my own filing date of November 2000, data were never presented by Fire, in the '559 specification or elsewhere, showing the effect of dsRNA on a vertebrate cell. And based upon the inventor's subsequent publications, he never tested to ascertain whether dsRNA could inhibit expression in a target gene in a vertebrate cell. While confirmation that the Fire '559 invention would actually function in a mammalian cell may not always be required of an inventor under U.S. Patent Law, some level of proof certainly would have been expected by one of ordinary skill in this art before accepting such a claim - since it was contrary to the recognized state of the art at the time of the Fire invention in 1998. At that time, and for several years thereafter, the expected response of post-embryonic mammalian cells, was known in the art to be very different from the operation of the '559 invention in an embryonic *C. elegans* cell. Yet the inventor only tested his invention using invertebrate cells. Fire certainly never made any

statement in the '559 specification or later, that would suggest that he believed that his invention would actually work in a vertebrate, mammalian or human cell. He simply included everything possible in his long boilerplate paragraphs – and even then preceded the list with a “may be.” Such lists certainly would not have led one of ordinary skill in 1997 or 1998 to attempt to practice the '559 invention in a vertebrate cell, because dsRNA inhibition of a target gene in a vertebrate cell was contrary to the response expected in such cells at the time, particularly when read in the context of published conflicting statements by the inventor himself about the effectiveness of the invention in mammalian cells. In fact, in the '559 specification, Fire failed to even identify the recognized and substantial defense mechanisms that one would have expected in a mammalian cell. Yet these defenses would have made the operation of dsRNA very different in such a mammalian cell, as compared to the '559 disclosed method of operation of dsRNA in an invertebrate embryonic cell.

9. Fire authored at least two papers (cited below) well after the effective filing date of the '559 patent, in which the very speculative nature of RNAi in mammalian cells, such as mice and humans, was made clear. Both have been previously provided to the Office.

10. In Montgomery and Fire, *TIG* 14:255-258 (1998), the authors discussed the inhibitory effect of dsRNA in embryonic *C. elegans* cells, and then ended the article with a discussion of their expected response of mammalian cells to dsRNA. Specifically, they note that a mammalian cell exposed to dsRNA “unleashes a vehement but somewhat non-specific response leading to general translational arrest” (page 258 sentence bridging columns 1 and 2). This phenomenon found in mammalian cells, refers to the effect of a cellular kinase known as PKR (protein kinase RNA-activated) which is induced by interferon and activated by RNA. The PKR response is one of several mammalian intracellular defense mechanisms, designed to inhibit viral infection, which operates by shutting down translation in a cell in the presence of long dsRNA, and ultimately leads to cell death by apoptosis. Fire and his co-author, thus speculated that “any gene-specific interference by dsRNA in PKR-proficient mammalian cells would be dependent on a transient lapse in the PKR response, or a controlled level of dsRNA that was incapable of activating PKR,” but how to accomplish either proposal was not mentioned in either the publication or in the earlier-filed patent.

11. Fire *et al.* offered neither their own teachings, nor pointed to any method known at the time for achieving such a lapse in the PKR response in post-embryonic, PKR-proficient, mammalian cells. Nor did they explain any reason why such expected defense mechanisms would not operate against the introduction of dsRNA into mammalian cells. In fact, the authors further indicated that, while there were suggestions in the literature of RNA-mediated interference in gene expression control, it remained unknown whether the RNA that induced such interference was single-stranded or double-stranded. See page 258, first full paragraph, second column. These statements clearly indicate that at that time in 1998, following the 1997 effective filing date of the application for the '559 patent, Fire himself recognized that any attempt to practice his invention as claimed in a mammalian cell, would be expected to result in a "vehement" PKR response that would render his method ineffective in any PKR-proficient mammalian cell, yet he had no idea how to overcome such a problem. Therefore, based upon the inventors' own statements, one skilled in the art would not have attempted to practice the '559 invention in a mammalian cell, since the recognized art at the time was that the PKR response and other mammalian defenses would violently block the effectiveness of dsRNA. As a result, based upon the '559 patent's teaching *only* of the response in embryonic invertebrate cells, in light of the mammalian defense mechanisms recognized in 1997 and 1998, such an individual would certainly not have expected dsRNA to induce RNA interference in a human cell, particularly since no data or explanation was provided by Fire regarding mammalian cell responses to dsRNA.

12. In *Trends Genet.* 15:358-363 (1999), Fire reiterated his concerns that his invention, as practiced in *C. elegans* embryonic cells, could not be practiced in a mammalian cell. On pp. 362-363 under "Real-world applications: what about us?" Fire states that "one *could* certainly *hope* that RNA-triggered silencing would exist in vertebrates" (emphasis added). Such a statement made at least a year after the 1997 effective filing date or 1998 actual filing date of the '559 patent, clearly shows that, at the filing date of the application for the '559 patent, Fire was not in possession of such information. As a result, Fire then continued in the 1999 paper that

"... the simple protocols used for invertebrates and plant systems *are unlikely to be effective* [in vertebrates]" (emphasis added).

13. In fact, Fire had no data or reason to believe that the expected PKR response or other intracellular defenses to foreign dsRNA could be overcome in a vertebrate cell. Accordingly, at

the filing date of the '559 patent (1998), one skilled in the art, *i.e.*, Fire himself, did not believe his claimed methods would work in mammalian cells. By 1999, Fire stated that he still did not believe that his invention could be applied to mammalian cells without substantial additional research, *i.e.*, it was “unlikely to be effective.” Consequently, it would certainly be accurate to say that such statements by Fire actually would have discouraged one of ordinary skill in the art from attempting to practice the '559 invention in vertebrate cells. Certainly, the 1999 publication taught one away from attempting to use dsRNA – in accordance with the method claimed in the '559 patent - in vertebrate or human cells.

14. A review of more recent literature confirms that as recently as 2001, neither Fire, nor anyone else, knew what response to expect from the introduction of dsRNA to a human cell. This was 4 years AFTER the effective filing date of the '559 patent application, and well after publication of the cited Fire manuscripts. However, what is clear is that the art did not believe that the Fire method would be effective in mammalian cells. Paddison *et al.*, *Proc. Nat'l Acad. Sci.* 99(3):1443-1448 (Feb. 5, 2002) (attached to show the subsequent state of the art) was presented and discussed at length with the Examiners in the July in-person interview. In the 2002 paper, Paddison *et al.* state (first and second columns, page 1443) that:

It has become clear that ds-RNA-induced silencing phenomena are present in evolutionarily diverse organisms including plants, fungi, and metazoans. . .

In several systems, dsRNA-induced silencing has been harnessed as a powerful tool for the analysis of gene function. Particularly in *Caenorhabditis elegans*, RNAi has emerged as the standard protocol for quickly assessing the consequences of inhibiting gene function. . . . In *Drosophila*, the first evidence of dsRNA-induced silencing came from the study of embryos, and insects. (citations omitted)

Standing alone, the preceding section would, like an unduly broad interpretation of the Fire patent, suggest that the teaching in *C. elegans* was the accepted standard for dsRNA to inhibit target gene function in *all* cells. But, in fact, when one continues to read Paddison *et al.* on page 1443, the authors clearly distinguish the art-recognized response of a vertebrate cell, from that of an invertebrate cell:

Despite its utility in diverse systems, harnessing RNA to study gene function in mammals seemed potentially problematic. Indeed, mammals have evolved robust systems for responding to dsRNAs . . .

Discussion then continues regarding mammalian intracellular defense mechanisms that are not found in the invertebrate cells that were tested by Fire in the '559 patent.

15. Then, in the Discussion section (first column, page 1448), Paddison *et al.* clearly state that:

The first indications that this response [to dsRNA] might also extend to mammals came from the observation that injections of dsRNAs into early mouse embryos induced sequence-specific silencing (23,24). Recent work by Tuschl and colleagues (5) had shown that siRNA can induce silencing in numerous mammalian cell lines . . . .

16. While the point of the discussion by Paddison *et al.* was directed to the transient nature of the gene silencing achieved by the early examination of the effect of dsRNA on mammals, several points are made clear. First, and foremost, the *first* researchers to publish results of dsRNA in a mammalian cell were Wianny *et al.*, *Nat. Cell Biol.* 2:70-75 (2000) (cited reference 23) and Svoboda *et al.*, *Development (Cambridge U.K.)* 127:4147-4156 (2000) (cited reference 24). Wianny *et al.* state in first column, page 71 that:

So far *there has been no report that RNAi can be used in mammals.* Moreover, there are several indications of potential limitations to its [dsRNA] function in this group of animals [mammals]. Principal among these is that the accumulation of very small amounts of dsRNA in mammalian cells following viral infection results in the interferon response, which leads to an overall block to translation and the onset of apoptosis. *Such considerations have discouraged investigators from using RNAi in mammals.* Emphasis added, cited references omitted.

17. Evidently, Fire's published work in invertebrate cells and the '559 patent, as well as his subsequent publications, are not even considered by other authors to be relevant to a discovery of the effect of dsRNA in mammalian cells. Moreover, the early work by Wianny *et al.* and by Svoboda *et al.*, each of which has been discussed at length in the recorded prosecution history of the present invention, relate to embryonic mammalian cells in which the defense mechanisms have not yet developed. Only the work by Tuschl *et al.* (cited as reference 5, Elbashir *et al.*, *Nature (London)* 411:494-498 (2001)) demonstrated the effect of dsRNA in mature mammalian cell lines – and that work was not reported until at least 5 months AFTER the effective filing date of my invention in November 2000.



18. The Examiner in the most recent Office Action, dated May 23, 2005, found our arguments to be unpersuasive. While acknowledging that the above-cited references recognized “potential difficulties in the application of dsRNA into mammalian cells,” the Examiner found that the art also provided “a reasonable means of overcoming said difficulties; *e.g.*; by using a controlled level of dsRNA.” However, I must strongly express my disagreement with such an argument for at least three reasons.

19. First, Fire made no mention in the ‘559 patent of the difficulties to be overcome in dealing with the intracellular defenses of mammalian cells. In fact, no difference was noted at all in the ‘559 patent between the expected response in a vertebrate cell, as compared with the data presented in an embryonic invertebrate cell – despite the fact that the state of the art at the time was well aware of the significant differences between the two, and of the defenses present in a viable mammalian cell. One could speculate that perhaps Fire was not aware of the differences. However, in a publication in 1998, he acknowledged that one would expect to encounter “vehement” defenses if dsRNA were introduced into a mammalian cell, and that such defenses would be expected to lead to translational arrest. In other words, Fire knew of the problems to be encountered, and expected that they would, indeed, probably block the silencing effect of the introduced dsRNA in a mammalian cell, yet he failed to identify the potential problem, nor did he offer any solution to the problem in the ‘559 specification. This is because neither he, nor anyone else, knew of a solution at the time of the filing date of the ‘559 invention. At that time, Fire simply was not in possession of an invention beyond the use of dsRNA in an embryonic invertebrate cell that would not encounter such intracellular defenses.

20. Second, while years after the filing date of the ‘559 patent, *others* clearly recognized problems associated with any attempt to apply the Fire methods (enabled only in embryonic invertebrate cells) to mammalian cells, and may have proposed solutions to the expected intracellular defenses - that is irrelevant to the rejection of my invention over the Fire patent. The fact is that Fire later acknowledged that use of his invention in vertebrate cells would encounter “vehement” defenses that would block the silencing of the target gene, yet he failed to address that anticipated problem in the ‘559 specification. The state of the art in 1997 and 1998 clearly showed that one of ordinary skill in the art would not have expected to use, or even tried to use, the ‘559 invention in a mammalian cell without being prepared to deal with the expected intracellular defenses. Fire recognized the problem, but offered no solution at the time of his

invention or later – presumably because he knew of no solution that would permit the use of his invention in mammalian cells. It would be improper and unfair to now credit Fire with an impermissibly broad interpretation of his claimed invention, extending far beyond what he enabled or possessed, to find my invention unpatentable – even though I accomplished what no one before me had done, or believed could be done – that is, to introduce dsRNA into a human cell and demonstrate the resulting disrupted expression of a target gene.

21. Third, the Examiners have said that my invention, a method for disrupting target gene expression at the mRNA level in a *human* cell, as defined in my above-identified claims, is unpatentable over the Fire invention because the ‘559 patent anticipated and taught every element of my invention before I did. If that were the case, then why was the work of Wianny *et al.* and Svoboda *et al.* considered “pioneering” in 2002 when others discussed the state of the art? Those researchers only dealt with embryonic mammalian cells in which the defense mechanisms have not yet developed, yet their work was considered a break-through because it addressed a problem that had not been previously solved in the art. It is clear that no one in 2002 recognized Fire’s work in 1997 or 1998 as sufficient to permit one of ordinary skill in the art to practice his claimed invention in a mammalian cell. If, as the Examiners have asserted, Fire provided sufficient disclosure in the ‘559 patent to permit the dsRNA silencing of a target gene in all cells – both invertebrate and vertebrate - then why did the art subsequently recognize Tuschl *et al.* for work in 2001 as being the *first* use of dsRNA in a mammalian cell line? In fact, prior to my work in human cells, and 5 months later as published by Tuschl – no one in the art was able to demonstrate the silencing effect of dsRNA on a target gene in a *human* cell. Accordingly, the answer to these questions is simple – Fire’s experiments in embryonic invertebrate cells simply did not, and could not, anticipate the target gene silencing effect of dsRNA in a human cell – regardless of the fact that claim 1 of the ‘559 patent failed to expressly state the understood species limitation to invertebrate cells.

22. The ‘559 claims do not expressly define the Fire invention as effective in a human cell. There is a good reason for what would otherwise be such an apparent oversight – Fire never believed that his invention would work in mammalian cells because he expected the intracellular defenses present in the mammalian cells to block translation of the foreign dsRNA. While the Fire patent may, in fact, be valid as to methods of treating cells of nematodes or invertebrate animals – the patent is simply not enabling for mammalian cells – nor could the patent expressly

claim a method that is effective in mammalian cells. The inventor never possessed such an invention – so he could not claim it or even describe how to overcome the expected problems. Fire never intended for his invention to actually be practiced in a vertebrate because he had no reason to believe that dsRNA could be introduced into viable mammalian cells. If the recognized problems were not already known at the time of the Fire invention, one might interpret the claims differently. But, to find that the Fire patent anticipated my invention would have required some teaching in either the '559 patent or in the prior art in 1997 or 1998, that would address the problem of intracellular defenses in a mammalian cell that were expected to attack the foreign dsRNA before it could act to silence the target gene. No such teaching was known or published AT THE FILING DATE OF THE FIRE '559 PATENT. The use of dsRNA in a mammalian cell was not discovered until years later. Paddison *et al.* were correct on page 1443, second column, when they stated that:

The ability to apply RNAi in mammals will undoubtedly spark a firestorm of effort to assess the consequences of suppressing the expression of genes in cultured mammalian cells.

23. When Paddison *et al.* made that statement in 2002, the Fire work (as claimed in the '559 patent) had been well published, yet even four years after the filing date of the Fire patent, no one had been able to apply RNAi in a mammalian cell, even though many were trying, and everyone wanted to succeed to place themselves at the forefront of the expected “firestorm” of success and recognition. The goal was clear, but actually silencing a mammalian gene with RNAi had proven far more elusive than the methods claimed by Fire in an invertebrate cell, or than the present Patent Examiners appear to have recognized.

24. The evidence is unmistakable when one reviews the history of RNAi; Fire's work was not the beginning of RNAi in mammals. While an important early piece of the puzzle, Fire's work could only teach what he had discovered in 1997 and 1998 – which was the use of dsRNA in an embryonic invertebrate cell that offered no intracellular defenses. Beyond that, since he could not solve the problem of intracellular defenses, Fire and his co-inventor could only speculate what might finally be discovered years later when investigators finally could apply RNAi to mammals.

25. Contrary to Fire *et al.*, the specification for my patent application offers no speculation. I have provided working examples showing that RNAi has actually been induced in CHP 100 neuro-epithelioma (melanoma) and HL-60 leukemia cells, which represent two different human cancer cell lines from different tissues and developmental origins. Because the instant application shows that RNAi can be induced in such widely divergent human cancer cell lines, one skilled in the art would understand that RNAi can now be induced in any human cell. Thus, the present specification (including the working examples) contains ample direction for how to practice the full breadth of my claimed methods.

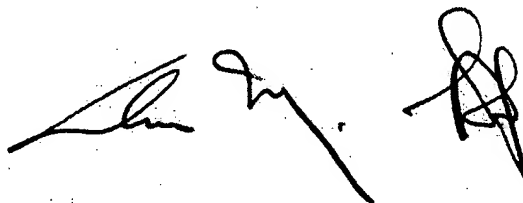
26. For example, the experiments described on page 12, line. 23 to page 14, line 6 of my present specification show that KitR expression was significantly reduced ( $p < 0.01$ ) in human cancer cells treated with 150 or 250  $\mu\text{g/ml}$  c-Kit dsRNA. However, no reduction in KitR expression was seen upon treatment of the cells with control green fluorescent protein (GFP) dsRNA. If the inhibition of KitR expression upon administration of c-Kit dsRNA had been due to a general inhibition of translation via the PKR response, then a similar reduction in KitR expression would have been induced by the GFP dsRNA. However, since KitR expression was reduced only upon administration of the target specific c-Kit dsRNA, the inhibition of KitR expression was necessarily due to gene-specific gene silencing (disrupted expression) as a result of the introduced dsRNA. Thus, the present specification, in describing and enabling the claimed invention, demonstrates the feasibility of inducing gene-specific RNAi in human cells with dsRNA, without apparent interference from the PKR response. This has been argued at length in the recorded prosecution history.

27. Until my work prior to November 2000 demonstrated the use of dsRNA to silence or disrupt the expression of a target gene in a *human* cell – no one – and I repeat – no one had, to my knowledge, been able to apply RNAi in mature human cells. I have always believed that the purpose of the US Patent System is to recognize the inventor who first discovers a new and highly useful invention. I have done that, and I am seeking only the fair and just patent recognition for what I have accomplished.

28. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patents issued thereon.

Respectfully submitted,

A handwritten signature in black ink, appearing to read 'Alan M. Gewirtz', with a stylized, looped flourish at the end.

Date: September 14, 2005

Alan M. Gewirtz

# Stable suppression of gene expression by RNAi in mammalian cells

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Communicated by Bruce W. Stillman, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, December 6, 2001 (received for review October 2, 2001)

In a diverse group of organisms including plants, *Caenorhabditis elegans*, *Drosophila*, and trypanosomes, double-stranded RNA (dsRNA) is a potent trigger of gene silencing. In several model systems, this natural response has been developed into a powerful tool for the investigation of gene function. Use of RNA interference (RNAi) as a genetic tool has recently been extended to mammalian cells, being inducible by treatment with small,  $\approx 22$ -nt RNAs that mimic those produced in the first step of the silencing process. Here, we show that some cultured murine cells specifically silence gene expression upon treatment with long dsRNAs ( $\approx 500$  nt). This response shows hallmarks of conventional RNAi including silencing at the posttranscriptional level and the endogenous production of  $\approx 22$ -nt small RNAs. Furthermore, enforced expression of long, hairpin dsRNAs induced stable gene silencing. The ability to create stable "knock-down" cell lines expands the utility of RNAi in mammalian cells by enabling examination of phenotypes that develop over long time periods and lays the groundwork for by using RNAi in phenotype-based, forward genetic selections.

The use of genetically tractable model systems has been the key to our present understanding of gene structure and function, cell and organismal biology, and, ultimately, the molecular aspects of human disease. The ability to stably knock out or knock down gene expression and, thus, function, in particular, has been paramount to the use of such models for illuminating biological function. For example, the use of conditional lethals in bacteriophage T4 allowed functional analysis of phage morphogenesis modules (1), whereas the same technique applied to yeast permitted the discovery of functional hierarchies among genes regulating cell cycle progression (2, 3). In both scenarios, cells acquire stable phenotypes through heritable genetic alterations.

Although such basic genetic approaches are virtually effortless in many model organisms, cultured mammalian cells have proven somewhat intractable, in this regard. This is largely because cultured mammalian cells are diploid and favor nonhomologous over homologous recombination. Current approaches to create stable phenotypes in mammalian cells have been often met with limited success. Dominant-negative and antisense strategies have proven inconsistent and unpredictable, thus lacking experimental rigor equivalent to a point mutation in yeast. However, one approach now used extensively in other diploid organisms has the potential to foment a revolution in mammalian somatic cell genetics. This approach is dubbed double-stranded RNA (dsRNA)-dependent posttranscriptional gene silencing, or RNA interference (RNAi).

It has become clear that dsRNA-induced silencing phenomena are present in evolutionarily diverse organisms including plants, fungi, and metazoans (reviewed in ref. 4). A combination of genetic and biochemical studies suggests that many of these phenomena share a common mechanism. The prevailing model begins with the conversion of the dsRNA silencing "trigger" into small RNAs (guide RNAs or siRNAs, ref. 5) that range in size from  $\approx 21$  to 25 nts, depending on the species of origin (6–8). These RNAs become incorporated into a multicomponent nuclease complex, which uses the sequence of the guide/siRNAs to identify and destroy homologous mRNAs (7, 8).

In several systems, dsRNA-induced silencing has been harnessed as a powerful tool for the analysis of gene function. Particularly in *Caenorhabditis elegans*, RNAi has emerged as the standard protocol for quickly assessing the consequences of inhibiting gene function. In fact, programs are underway to create RNAi libraries that can be used to suppress, individually, each of the  $\approx 19,000$  genes in the worm genome (9, 10). In *Drosophila*, the first evidence of dsRNA-induced silencing came from the study of embryos (11), and subsequently, RNAi has proven an effective tool in cultured cells and in adult insects (7, 12, 13).

Despite its utility in diverse systems, harnessing RNA to study gene function in mammals seemed potentially problematic. Indeed, mammals have evolved robust systems for responding to dsRNAs, specifically as an antiviral defense (reviewed in refs. 14 and 15). In somatic cells, dsRNA activates a variety of responses. Predominant among these is PKR, a kinase that is activated by dimerization in the presence of dsRNA (16). PKR, in turn, phosphorylates EIF2 $\alpha$ , causing a nonspecific translational shutdown (reviewed in ref. 14). dsRNA also activates 2'-5' oligoadenylate polymerase, the product of which is an essential cofactor for a nonspecific ribonuclease, RNase L (reviewed in ref. 17).

Recently, Tuschl and colleagues (5) have demonstrated that RNAi can be provoked in numerous mammalian cell lines through the introduction of siRNAs. These siRNAs avoid provoking the PKR response by virtue of their small size and are presumed to be incorporated into the RNAi pathway by mimicking the products of the Dicer enzyme, which catalyzes the initiation step of RNAi (18). The ability to apply RNAi in mammals will undoubtedly spark a firestorm of effort to assess the consequences of suppressing the expression of genes in cultured mammalian cells.

The power of RNAi as a genetic tool would be greatly enhanced by the ability to engineer stable silencing of gene expression. Whereas the production of small RNAs via *in vivo* expression is problematic, stable silencing has been induced in model organisms by directed expression of long dsRNAs (13, 19, 20). We therefore undertook an effort to identify mammalian cells in which long dsRNAs could be used as RNAi triggers in the hope that these same cell lines would provide a platform upon which to develop stable silencing strategies.

## Materials and Methods

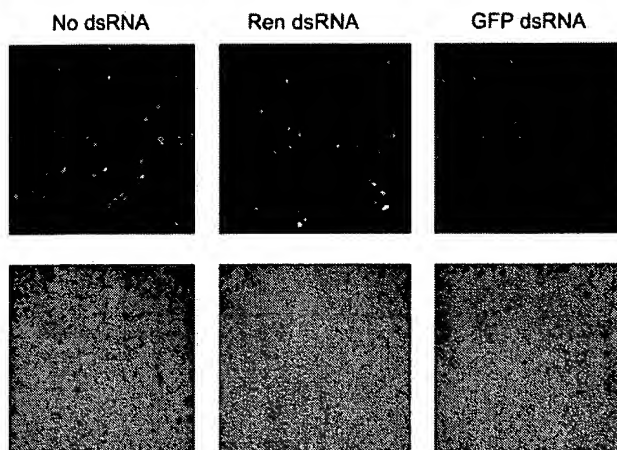
**Cell Culture.** P19 mouse embryonic carcinoma cells (American Type Culture Collection, CRL-1825) were cultured in  $\alpha$ -MEM (GIBCO/BRL) supplemented with 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution (GIBCO/BRL). Mouse embryo stem cells (J1, provided by S. Kim, Cold Spring Harbor Laboratory) were cultured in DMEM containing ESgro (Chemicon) according to the manufacturer's instructions. C2C12 murine myoblast cells (gift of N. Tonks, Cold Spring Harbor

Abbreviations: dsRNA, double-stranded RNA; RNAi, RNA interference; siRNA, small interfering RNA; EGFP, enhanced green fluorescent protein.

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**Fig. 1.** RNAi in P19 embryonal carcinoma cells. Ten-centimeter plates of P19 cells were transfected by using 5  $\mu$ g of GFP plasmid and 40  $\mu$ g of the indicated dsRNA (or no RNA). Cells were photographed by fluorescent and phase-contrast microscopy at 72 h after transfection; silencing was also clearly evident at 48 h posttransfection.

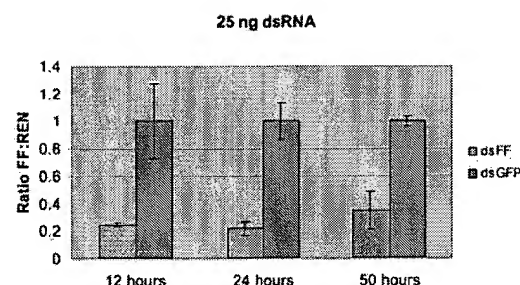
Laboratory) were cultured in DMEM (GIBCO/BRL) supplemented with 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution (GIBCO/BRL).

**RNA Preparation.** For the production of dsRNA, transcription templates were generated by PCR; they contained T7 promoter sequences on each end of the template (see ref. 7). dsRNAs were prepared by using the RiboMax kit (Ambion, Austin, TX). Firefly and *Renilla* luciferase mRNA transcripts were synthesized by using the Riboprobe kit (Promega) and were gel purified before use.

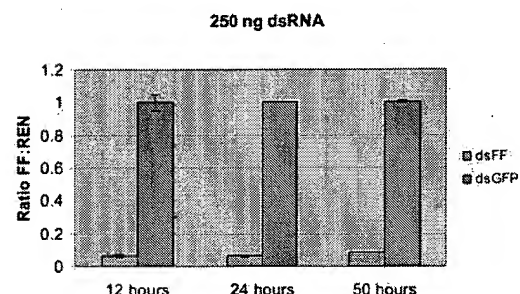
**Transfection and Gene Silencing Assays.** Cells were transfected with indicated amounts of dsRNA and plasmid DNA by using FuGENE6 (Roche Biochemicals) according to the manufacturer's instructions. Cells were transfected at 50–70% confluence in 12-well plates containing either 1 or 2 ml of medium per well. Dual luciferase assays (Promega) were carried out by cotransfecting cells with plasmids contain firefly luciferase under the control of SV40 promoter (pGL3-Control, Promega) and *Renilla* luciferase under the control of the SV40 early enhancer/promoter region (pSV40, Promega). These plasmids were cotransfected by using a 1:1 or 10:1 ratio of pGL3-control (250 ng/well) to pRL-SV40. Both ratios yielded similar results. For some experiments, cells were transfected with vectors that direct expression of enhanced green fluorescent protein (EGFP)-US9 fusion protein (21) or red fluorescent protein (pDsRed N1, CLONTECH). RNAi in S2 cells was performed as described (7).

Plasmids expressing hairpin RNAs (RNAs with a self-complementary stem loop) were constructed by cloning the first 500 bp of the EGFP coding region (CLONTECH) into the FLIP cassette of pRIP-FLIP (E. Bernstein and G.J.H., unpublished data) as a direct repeat. The FLIP cassette contains two directional cloning sites, the second of which sports flanking LoxP sites (see Fig. 6A). The Zeocin gene (Stratagene), present between the cloning sites, maintains selection and, thus, stability of the FLIP cassette. The FLIP cassette containing EGFP direct repeats was subcloned into pcDNA3 (Invitrogen). To create an inverted repeat for hairpin production, EGFP direct repeat clones were exposed to Cre recombinase (Stratagene) *in vitro* and, afterward, transformed into DL759 *Escherichia coli* (22). These bacteria permit the replication of DNA containing cruciform structures, which tend to form from inverted repeats.

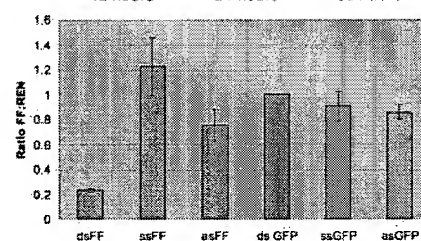
**A.**



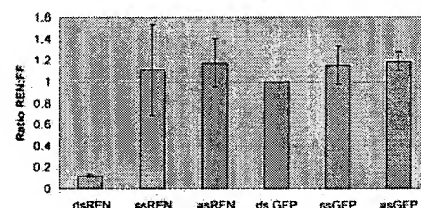
**B.**



**C.**



**D.**

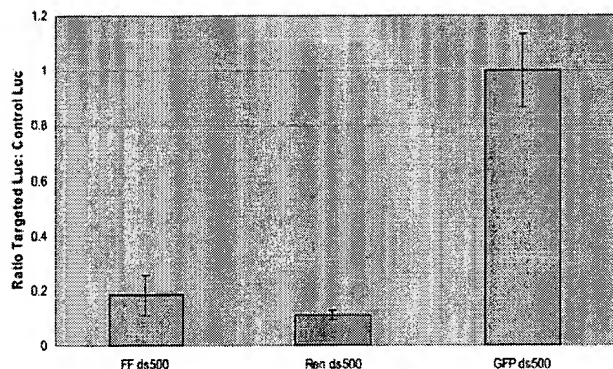


**Fig. 2.** RNAi of firefly and *Renilla* luciferase in P19 cells. (A) P19 cells were transfected with plasmids that direct the expression of firefly and *Renilla* luciferase and dsRNA 500 mers (25 or 250 ng, as indicated), that were either homologous to the firefly luciferase mRNA (dsFF) or nonhomologous (dsGFP). Luciferase activities were assayed at various times after transfection, as indicated. Ratios of firefly to *Renilla* activity are normalized to dsGFP controls. (B and C) P19 cells in 12-well culture dishes (2 ml of media) were transfected with 0.25  $\mu$ g of a 9:1 mix of pGL3-Control and pRL-SV40 as well as 2  $\mu$ g of the indicated RNA. Extracts were prepared 9 h after transfection. (B) Ratio of firefly to *Renilla* luciferase is shown. (C) Ratio of *Renilla* to firefly luciferase is shown. Values are normalized to dsGFP. The average of three independent experiments is shown; error bars indicate standard deviation.

DL759 transformants were screened for plasmids containing inverted repeats ( $\approx 50\%$ ).

Silencing of Dicer was accomplished by using a dsRNA comprising exon 25 of the mouse Dicer gene and corresponding to nucleotides 5284–5552 of the human Dicer cDNA.

**In Vitro Translation and in Vitro Dicer Assays.** Logarithmically growing cells were harvested in PBS containing 5 mM EGTA washed twice in PBS and once in hypotonic buffer (10 mM Hepes, pH 7.3/6 mM  $\beta$ -mercaptoethanol). Cells were suspended in 0.7 packed-cell



**Fig. 3.** Specific silencing of luciferase expression by dsRNA in murine embryonic stem cells. Mouse embryonic stem cells in 12-well culture dishes (1 ml of media) were transfected with 1.5  $\mu$ g of dsRNA along with 0.25  $\mu$ g of a 10:1 mixture of the reporter plasmids pGL3-Control and pRL-SV40. Extracts were prepared and assayed 20 h after transfection. The ratio of firefly to *Renilla* luciferase expression is shown for FF ds500; the ratio of *Renilla* to firefly is shown for Ren ds500. Both are normalized to ratios from the dsGFP transfection. The average of three independent experiments is shown; error bars indicate standard deviation.

volumes of hypotonic buffer containing *Complete* protease inhibitors (Roche Molecular Biochemicals) and 0.5 units/ml of RNasin (Promega). Cells were disrupted in a Dounce homogenizer with a type B pestle, and lysates were centrifuged at  $30,000 \times g$  for 20 min. Supernatants were used in an *in vitro* translation assay containing capped m7G(5')pppG firefly and *Renilla* luciferase mRNA or in *in vitro* Dicer assays containing  $^{32}$ P-labeled dsRNA. For *in vitro* translation assays, 5  $\mu$ l of extract were mixed with 100 ng of firefly and *Renilla* mRNA along with 1  $\mu$ g of dsRNA (or buffer)/10 mM DTT/0.5 mM spermidine/200 mM Hepes, 3.3 mM MgOAc/800 mM KOAc/1 mM ATP/1 mM GTP/4 units of Rnasin/215  $\mu$ g of creatine phosphate/1  $\mu$ g of creatine phosphate kinase/1 mM amino acids (Promega). Reactions were carried out for 1 h at 30°C and quenched by adding  $1 \times$  passive lysis buffer (Promega). Extracts were then assayed for luciferase activity. *In vitro* assays for Dicer activity were performed as described (18).

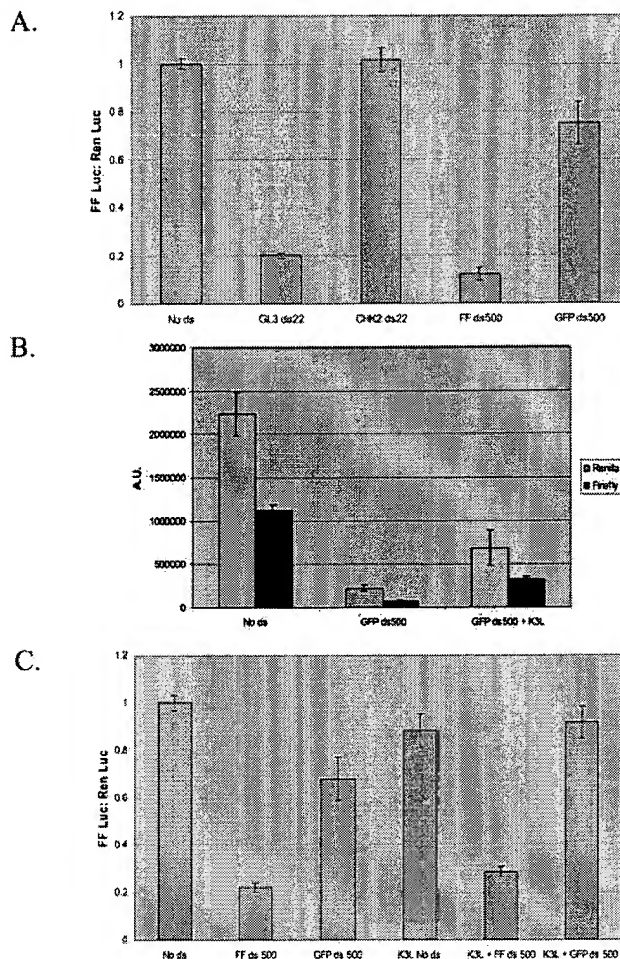
**Construction of Stable Silencing Lines.** Ten-centimeter plates of P19 cells were transfected with 5  $\mu$ g of GFP hairpin expression plasmid and selected for stable integrants by using G-418 (300 ng/ml) for 14 days. Clones were selected and screened for silencing of GFP.

## Results

**RNAi in Pluripotent Murine P19 Cells.** It has long been clear that the nonspecific responses to dsRNA are attenuated during early development. In fact, injection of dsRNA into early-stage mouse embryos can induce sequence-specific silencing of both exogenous and endogenous genes (23, 24). Consistent with the possibility that RNAi might extend to mammals, homologs of the proteins that participate in this response can be easily identified in the mouse and human genomes (reviewed in ref. 4).

We sought to determine whether long dsRNA triggers could induce sequence-specific silencing in cultured murine cells, both to develop this approach as a tool for probing gene function and to allow mechanistic studies of dsRNA-induced silencing to be propagated to mammalian systems. We, therefore, attempted to extend previous studies in mouse embryos (23, 24) by searching for RNAi-like mechanisms in pluripotent, embryonic cell types.

We surveyed a number of cell lines of embryonic origin for the degree to which generalized suppression of gene expression occurred upon introduction of dsRNA. As an assay, we tested the

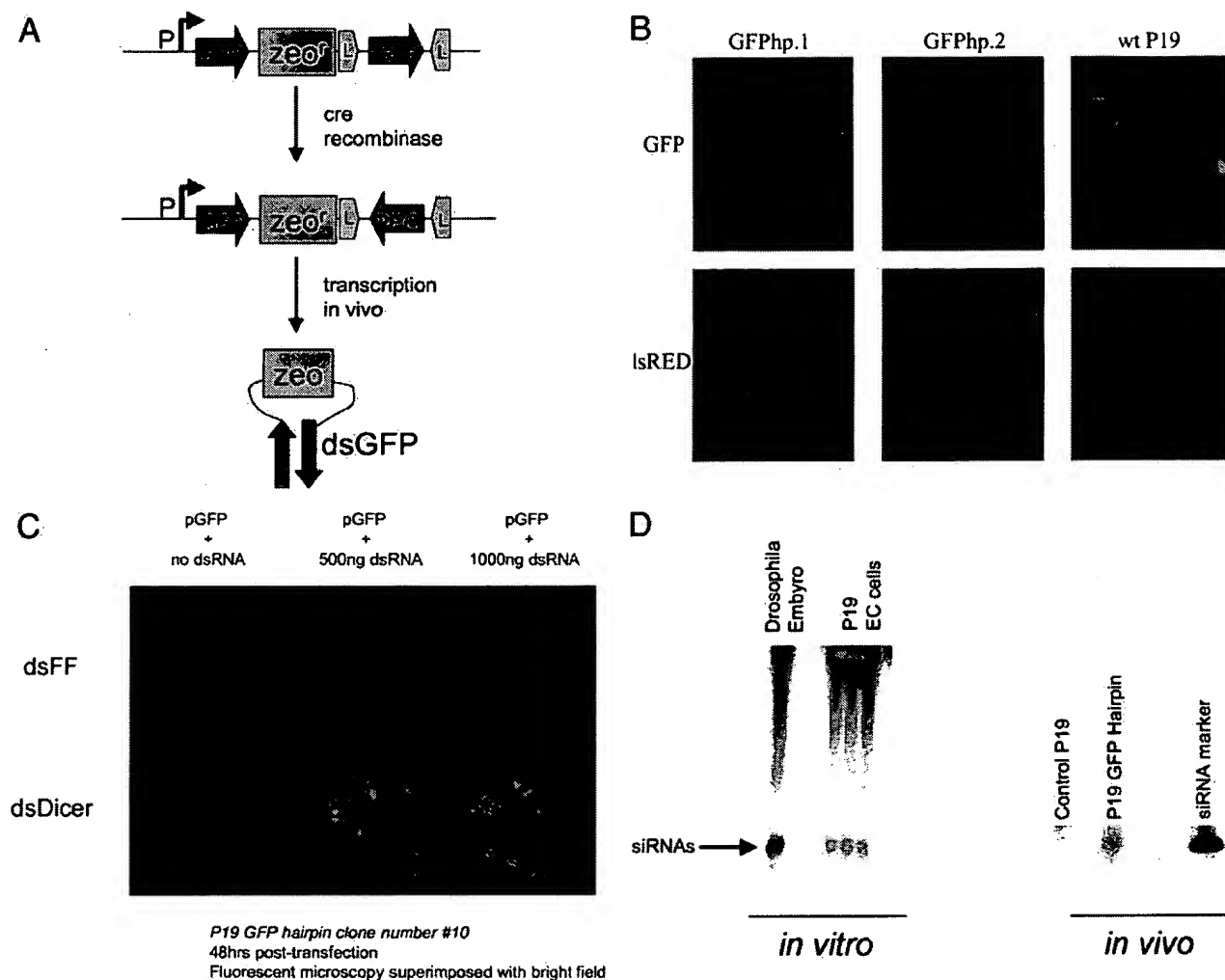


**Fig. 4.** RNAi in C2C12 murine myoblast cells. (A) Mouse C2C12 cells in 12-well culture dishes (1 ml of media) were transfected with 1  $\mu$ g of the indicated dsRNA along with 0.25  $\mu$ g of the reporter plasmids pGL3-Control and pRL-SV40. Extracts were prepared and assayed 24 h after transfection. The ratio of firefly to *Renilla* luciferase expression is shown; values are normalized to ratios from the no dsRNA control. The average of three independent experiments is shown; error bars indicate standard deviation. (B) C2C12 cells cotransfected with 1  $\mu$ g of either plasmid alone or a plasmid containing a hyperactive mutant of vaccinia virus K3L (26). The absolute counts of *Renilla* and firefly luciferase activity are shown. (C) The ratios of firefly/*Renilla* activity from B, normalized to no dsRNA controls.

effects of dsRNA on the expression of GFP as measured *in situ* by counting fluorescent cells. As expected, in both human embryonic kidney cells (293) and mouse embryo fibroblasts, GFP expression was virtually eliminated irrespective of the sequence of the cotransfected dsRNA (not shown). In some pluripotent teratocarcinoma and teratoma cell lines (e.g., N-Tera1, F9), the PKR response was attenuated but still evident (not shown); however, in contrast, transfection of nonhomologous dsRNAs had no effect on the expression of reporter genes (e.g., GFP, luciferase) either in mouse embryonic stem cells (not shown) or in p19 embryonal carcinoma cells (Fig. 1).

Transfection of P19 embryonal carcinoma cells with GFP in the presence of cognate dsRNA corresponding to the first  $\sim 500$  nts of the GFP coding sequence had a strikingly different effect. GFP expression was eliminated in the vast majority of cotransfected cells (Fig. 1), suggesting that these cultured murine cells might respond





**Fig. 5.** Expression of a hairpin RNA produces P19 EC cell lines that stably silence GFP. (A) A cartoon of the FLIP cassette used to construct the GFP hairpin. GFP represents the first 500 coding base pairs of EGFP. Zeo, zeocin resistance gene; L, Lox; P, the cytomegalovirus promoter in the expression plasmid pCDNA3. Homologous GFP fragments are first cloned as direct repeats into the FLIP cassette. To create inverted repeats for hairpin production, the second repeat is flipped by using Cre recombinase. When transcribed, the inverted repeat forms a GFP dsRNA with a hairpin loop. (B) P19 cell lines stably expressing the GFP hairpin plasmid, GFP<sub>hp</sub>.1 (clone 10) and GFP<sub>hp</sub>.2 (clone 12), along with wt P19 were transfected with 0.25  $\mu$ g each of GFP and RFP reporter genes. Fluorescence micrographs were taken by using filters appropriate for GFP and RFP. Magnification is 200 $\times$ . (C) P19 GFP<sub>hp</sub>.1 cells were transfected with pEGFP and 0, 0.5, or 1  $\mu$ g of Dicer or firefly dsRNA. Fluorescence micrographs were taken at 48 h posttransfection and are superimposed with bright field images to reveal non-GFP expressing cells. Magnification is 100 $\times$ . (D) *In vitro* and *in vivo* processing of dsRNA in P19 cells. *In vitro* Dicer assays were performed on S2 cells and three independently prepared P19 extracts by using  $^{32}$ P-labeled dsRNA (30 $^{\circ}$ C for 30 min). A Northern blot of RNA extracted from control and GFP<sub>hp</sub>.1 P19 cells shows the production of  $\sim$ 22mer RNA species in hairpin-expressing cells but not in control cells. Blots were probed with a  $^{32}$ P-labeled "sense" GFP transcript.

to dsRNA in a manner similar to that which we had previously demonstrated in cultured, *Drosophila* S2 cells (7).

To quantify the extent to which dsRNA could induce sequence-specific gene silencing, we used a dual luciferase reporter assay similar to that which had first been used to demonstrate RNAi in *Drosophila* embryo extracts (25). P19 EC cells were transfected with a mixture of two plasmids that individually direct the expression of firefly luciferase and *Renilla* luciferase. These were cotransfected with no dsRNA, with dsRNA that corresponds to the first  $\sim$ 500 nts of the firefly luciferase, or with dsRNA corresponding to the first  $\sim$ 500 nts of GFP as a control. Cotransfection with GFP dsRNA gave luciferase activities that were similar to the no-dsRNA control, both in the firefly/*Renilla* activity ratio and in the absolute values of both activities. In contrast, in cells that received the firefly luciferase dsRNA, the ratio of firefly to *Renilla* luciferase activity

was reduced by up to 30-fold (250 ng, Fig. 2B). For comparison, we carried out an identical set of experiments in *Drosophila* S2 cells. Although qualitatively similar results were obtained, the silencing response was more potent. At equivalent levels of dsRNA, S2 cells suppressed firefly luciferase activity to virtually background levels (not shown).

The complementary experiment, in which dsRNA was homologous to *Renilla* luciferase, was also performed. Again, in this case, suppression of the expression of the *Renilla* enzyme was  $\sim$ 10-fold (Fig. 2D). Thus, the dsRNA response in P19 cells was flexible, and the silencing machinery was able to adapt to dsRNAs directed against any of the reporters that were tested.

We took two approaches to test whether this response was specific for dsRNA. Pretreatment of the trigger with purified RNase III, a dsRNA-specific ribonuclease, before transfection

greatly reduced its ability to provoke silencing (not shown). Furthermore, transfection of cells with single-stranded antisense RNAs directed against either firefly or *Renilla* luciferase had little or no effect on expression of the reporters (Fig. 2 C and D). Considered together, these results provided a strong indication that double-stranded RNAs provoke a potent and specific silencing response in P19 embryonal carcinoma cells.

Efficient silencing could be provoked with relatively low concentrations of dsRNA (25 ng/ml culture media; see Fig. 2A). The response was concentration-dependent, with maximal suppression of  $\approx 20$ -fold being achieved at a dose of 1.5  $\mu\text{g}/\text{ml}$  culture media.

Silencing was established rapidly and was evident by 9 h post-transfection (the earliest time point examined). Furthermore, the response persisted without significant changes in the degree of suppression for up to 72 h following a single dose of dsRNA.

**RNAi in Embryonic Stem Cells.** To assess whether the presence of a sequence-specific response to dsRNA was a peculiarity of P19 cells or whether it also extended to normal murine embryonic cells, we performed similar silencing assays in mouse embryonic stem cells. Cotransfection of embryonic stem cells with noncognate dsRNAs (e.g., GFP), again, had no dramatic effect on either the absolute values or the ratios of *Renilla* and firefly luciferase activity (Fig. 3). However, transfection with either firefly or *Renilla* luciferase dsRNA dramatically and specifically reduced the activity of the targeted enzyme (Fig. 3).

This result suggests that RNAi can operate in multiple murine cell types of embryonic origin, including normal embryonic stem cells. The ability to provoke silencing in a cell type that is normally used for the generation of genetic, mosaic animals suggests the possibility of eventually testing the biological effects of silencing both in culture and in reconstituted animal models.

**RNAi in Murine Somatic Cells.** RNAi effector pathways are likely to be present in mammalian somatic cells, based on the ability of siRNAs to induce transient silencing (5). Furthermore, we have shown that RNAi initiator and effector pathways clearly exist in embryonic cells that can enforce silencing in response to long dsRNA triggers. We therefore sought to test whether the RNAi machinery might exist intact in some somatic cell lines.

Transfection of HeLa cells with luciferase reporters in combination with long dsRNA triggers caused a nearly complete suppression of activity, irrespective of the RNA sequence. In a murine myoblast cell line, C2C12, we noted a mixture of two responses. dsRNAs homologous to firefly luciferase provoked a sequence-specific effect, producing a degree of suppression that was slightly more potent than was observed upon transfection with cognate  $\approx 21$ -nt siRNA (ref. 5; Fig. 4A). However, with long dsRNA triggers, the specific effect was superimposed upon a generalized suppression of reporter gene expression that was presumably because of PKR activation (Fig. 4B).

Numerous mammalian viruses have evolved the ability to block PKR as an aid to efficient infection. For example, adenoviruses express VA RNAs, which mimic dsRNA with respect to binding but not to activation of PKR (16). Vaccinia virus uses two strategies to evade PKR. First is expression of E3L, which binds and masks dsRNAs (26). The second is expression of K3L, which binds and inhibits PKR via its ability to mimic the natural substrate of this enzyme, eIF2 $\alpha$  (26).

Transfection of C2C12 cells with a vector that directs K3L expression attenuates the generalized repression of reporter genes in response to dsRNA. However, this protein had no effect on the magnitude of specific inhibition by RNAi (Fig. 4C).

These results raise the possibility that, at least in some cell lines and/or cell types, blocking nonspecific responses to dsRNA will enable the use of long dsRNAs for the study of gene function. This might be accomplished through the use of viral inhibitors, as

described here, or through the use of cells isolated from animals that are genetically modified to lack undesirable responses.

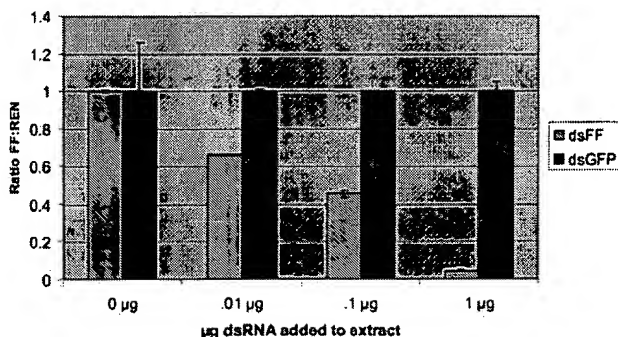
**Stable Suppression of Gene Expression Using RNAi.** To date, dsRNAs have been used to induce sequence-specific gene silencing in either cultured mammalian cells or in embryos only in a transient fashion. However, the most powerful applications of genetic manipulation are realized only with the creation of stable mutants. The ability to induce silencing by using long dsRNAs offers the opportunity to translate into mammalian cells work from model systems such as *Drosophila*, plants, and *C. elegans* wherein stable silencing has been achieved by enforced expression of hairpin RNAs (13, 19, 20).

P19 EC cells were transfected with a control vector or with an expression vector that directs expression of a  $\approx 500$ -nt GFP hairpin RNA from an RNA polymerase II promoter (cytomegalovirus). Colonies arising from cells that had stably integrated either construct were selected and expanded into clonal cell lines. Each cell line was assayed for persistent RNAi by transient cotransfection with a mixture of two reporter genes, dsRED to mark transfected cells and GFP to test for stable silencing.

Transfection of clonal P19 EC cells that had stably integrated the control vector produced equal numbers of red and green cells, as would be expected in the absence of any specific silencing response (Fig. 5B), whereas cells that express the GFP hairpin RNA gave a very different result. These cells expressed the dsRED protein with an efficiency comparable to that observed in cells containing the control vector. However, the cells failed to express the cotransfected GFP reporter (Fig. 5B). These data provide a strong indication that continuous expression of a hairpin dsRNA can provoke stable, sequence-specific silencing of a target gene.

In *Drosophila* S2 cells and *C. elegans* (18, 27–30), RNAi is initiated by the Dicer enzyme, which processes dsRNA into  $\approx 22$ -nt siRNAs (18). In both, S2 cells and *C. elegans* experiments by using dsRNA to target Dicer suppress the RNAi response (18, 27, 29). Whether Dicer plays a central role in hairpin-induced gene silencing in P19 cells was tested by transfecting P19 cells stably transfected with GFP hairpin constructs with mouse *Dicer* dsRNA (see *Materials and Methods*). Treatment with *Dicer* dsRNA, but not control dsRNA, resulted in derepression of GFP (Fig. 5C).

**dsRNA Induces Posttranscriptional Silencing.** A key feature of RNAi is that it exerts its effect at the posttranscriptional level by destruction of targeted mRNAs (reviewed in ref. 4). To test



**Fig. 6.** dsRNA induces silencing at the posttranscriptional level. P19 cell extracts were used for *in vitro* translation of firefly and *Renilla* luciferase mRNA (100 ng each). Translation reactions were programmed with various amounts of dsRNA 500mers, either homologous to firefly luciferase mRNA (dsLUC) or nonhomologous (dsGFP). Luciferase assays were carried out after a 1-h incubation at 30°C. Ratios of firefly to *Renilla* activity are normalized to no dsRNA controls. Standard deviations from the mean are shown.

whether dsRNAs induced silencing in mouse cells via posttranscriptional mechanisms, we used an assay identical to that, used initially to characterize RNAi responses in *Drosophila* embryo extracts (25). We prepared lysates from P19 EC cells that were competent for *in vitro* translation of capped mRNAs corresponding to *Renilla* and firefly luciferase. Addition of nonspecific dsRNAs to these extracts had no substantial effect on either the absolute amount of luciferase expression or on the ratio of firefly to *Renilla* luciferase (Fig. 6). In contrast, addition of dsRNA homologous to the firefly luciferase induced a dramatic and dose-dependent suppression of activity. Addition of RNA corresponding to only the antisense strand of the dsRNA had little effect, comparable to a nonspecific dsRNA control, and pretreatment of the dsRNA silencing trigger with RNase III greatly reduced its potential to induce silencing *in vitro*. A second hallmark of RNAi is the production of small,  $\approx 22$ -nt siRNAs, which determine the specificity of silencing. We found that such RNA species were generated from dsRNA in P19 cell extracts (Fig. 5D, *in vitro*), indicative of the presence of a mouse Dicer activity. These species were also produced in cells that stably express GFP hairpin RNAs (Fig. 5D, *in vivo*). Considered together, the posttranscriptional nature of dsRNA-induced silencing, the association of silencing with the production of  $\approx 22$ -nt siRNAs, and the dependence of this response on Dicer, a key player in the RNAi pathway, strongly suggests that dsRNA suppresses gene expression in murine cells via a conventional RNAi mechanism.

## Discussion

The discovery that dsRNA could induce gene silencing in organisms as diverse as plants and parasitic protozoans has raised the possibility that RNAi might be a nearly universal mechanism of gene silencing. This notion has been supported by the identification of homologs of proteins that participate in the silencing process in virtually all genomes examined to date, with the exception of *Saccharomyces cerevisiae* (reviewed in ref. 4). The first indications that this response might also extend to mammals came from the observation that injection of dsRNAs into early mouse embryos induced sequence-specific silencing (23, 24). Recent work by Tuschl and colleagues (5) had shown that siRNAs can induce silencing in numerous mammalian cell lines, presumably by entering the RNAi pathway. However, both in mouse embryos and previous mammalian cell culture studies, silencing was transient.

As an extension of these pioneering studies, we have demonstrated that dsRNA can induce potent and specific gene silencing in mouse embryonic cell lines. Specifically, we have shown that silencing can be induced by long dsRNAs in mouse embryonic carcinoma cell lines, in normal mouse embryonic stem cells, and in some mouse somatic cells. There are several indications that this phenomenon might be mechanistically related to RNA interference pathways that have been characterized in plants, *C. elegans*, and *Drosophila*. First, induction of silencing requires dsRNA. Second, *in vitro* studies suggest that silencing occurs at the posttranscriptional level. Third, silencing is correlated with the appearance of  $\approx 22$ -nt siRNAs homologous to the gene that is being suppressed. However, final placement of the phenomenon reported here within the pantheon of dsRNA-induced silencing mechanisms will require a characterization of the protein and/or ribonucleoprotein machinery, which enforces suppression. A significant step toward this goal has been taken by the demonstration that Dicer is required for dsRNA-induced silencing in P19 cells.

We have demonstrated that stable, sequence-specific silencing can be induced by enforcing endogenous expression of RNA hairpins. The ability to create permanent cell lines with a desired loss-of-function phenotype extends the utility of RNAi as method for probing gene function in mammalian cells. This capability enables the production of large numbers of silenced cells for biochemical analysis and permits the evaluation of phenotypes over long time spans. However, perhaps the two most important ramifications of stable RNAi are the ability to harness this technology for unbiased, phenotype-based genetic selections and the possibility that stably silenced, embryonic cell lines might ultimately be used to reconstitute animals containing a specifically silenced locus.

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